Articles

Dynamic Conformations Compared for IgE and IgG1 in Solution and Bound to Receptors†

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ABSTRACT: Dynamic conformations of two distinct immunoglobulin (Ig) isotypes, murine IgE and human IgG1, were examined with fluorescence resonance energy transfer measurements. The IgE mutant $\epsilon/C\gamma3^*$ and the IgG1 mutant $\gamma/C\gamma3^*$ each bind [5-(dimethylamino)naphthalen-1-yl]sulfonyl (DNS) in two identical antigen binding sites at the amino (N)-terminal ends of the Ig in the Fab segments. Eosin–DNS bound in these Fab sites served as the acceptor probe in these studies. Both Ig have a carboxy (C)-terminal domain ($C\gamma3^*$) which contains genetically introduced cysteine residues. Modification of these cysteine sulfhydryls with fluorescein maleimide provided donor probes near the C-terminal ends of the Ig in the Fc segment. Energy transfer between the C-terminal and N-terminal ends was compared for these two Ig in solution and when they were found to their respective high-affinity receptors on plasma membranes: IgE–FcϵRI on RBL cell membranes and IgG1–FcγRI on U937 cell membranes. Previous energy-transfer measurements with these probes yielded an average end-to-end distance of 71 Å for IgE in solution and 69 Å for IgE bound to FcϵRI, indicating that in both situations IgE is bent such that the axes of the Fab segments and the axis of the Fc segment do not form a planar Y-shape [Zheng, Shopes, Holowka, & Baird (1991) *Biochemistry 30*, 9125]. In the current study we found the average end-to-end distance for IgG1 in solution is 75 Å and ≥85 Å for IgG1 bound to FcγRI, suggesting an average bend conformation for IgG1 as well. The contributions of segmental flexibility to the average distances were assessed directly by measuring the efficiency of energy transfer as a function of variations in donor quantum yield caused by a collisional quencher and using these data to extract a Gaussian distribution of end-to-end distances. The distribution average (ρ) and half-width (hw) were determined to be as follows: ρ = 75 Å, hw = 24 Å for IgE in solution; ρ = 71 Å, hw = 12 Å for IgE bound to FcϵRI; and ρ = 100 Å, hw = 88 Å for IgG in solution. All of the results obtained are consistent with the view that IgE bound to FcϵRI is bent with limited motion between N-terminal and C-terminal ends and that the structure is similar, although somewhat less rigid, for IgE in solution. In contrast, IgG in solution appears to be much more flexible, and this flexibility can account for the short average end-to-end distance measured.

Ig[1] molecules recognize diverse foreign antigens and trigger a variety of immune responses. This is achieved by the linkage of two Fab segments, which bind to antigens, with an Fc segment, which interacts with some type of effector molecule. The conformation of the Ig and the flexibility of the Fab and Fc segments about their linking region are probably important for function (Burton, 1990). Within the IgG class the Fab and Fc segments are linked by a hinge consisting of extended segments of the γ chains between the $C\gamma1$ and $C\gamma2/C\gamma3$ domains. The length and amino acid sequence of this hinge vary markedly among the members of the IgG subclasses, and this correlates with differences in Fab segmental flexibility (Oi et al., 1984; Dangl et al., 1988). In IgE the ϵ heavy chain contains an extra domain, $C\epsilon2$, in the region corresponding to the hinge of IgG. The $C\epsilon3/C\epsilon4$ domains are homologous to the $C\gamma2/C\gamma3$ domains, respectively, of IgG. In accordance with these structural differences in the "hinge region", IgE in solution appears to have less Fab segmental flexibility than the IgG subclasses as revealed by fluorescence anisotropy measurements (Oi et al., 1984). IgE binds to its high-affinity cell surface receptor (FcϵRI) in the region of the $C\epsilon3$ domain or the $C\epsilon2/C\epsilon3$ interface (Helm et al., 1988; Weetall et al., 1990; Nissim et al., 1991). When receptor-bound, the Fab segmental flexibility of IgE is clearly limited, although a relatively fast component can be detected (Slattery et al., 1985; Holowka et al., 1990).

Electron micrographs of murine IgG1 bound to influenza hemagglutinin provide evidence for substantial flexibility of the Fab and Fc segments about the hinge region (Wrigley et al., 1983; Phillips et al., 1990). Two-dimensional ordered

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[1] Abbreviations: Ig, immunoglobulin(s); $C\gamma3$, carboxy-terminal domain of IgG heavy chain (γ); $C\epsilon4$, carboxy-terminal domain of IgE heavy chain (ϵ); $\gamma/C\gamma3^*$, variant of IgG1 with cysteine genetically introduced into $C\gamma3$ domain near the carboxy terminus; $\epsilon/C\gamma3$, variant of IgE with $C\epsilon4$ replaced by $C\gamma3$; $\epsilon/C\gamma3^*$, variant of IgE with $C\epsilon4$ replaced by $C\gamma3^*$ containing genetically introduced cysteine near the carboxy terminus; DNS, [5-(dimethylamino)naphthalen-1-yl]sulfonyl; DNS-Lys, N^{ϵ}-DNS-L-lysine; DTT, dithiothreitol; ED, 1-*N*-(eosinthiocarbamyl)-5-*N*-DNS-cadaverine; FM, fluorescein-5-maleimide; PBS, phosphate-buffered saline.

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arrays of murine IgG1 and IgE bound, via their Fab segments, to haptenated phospholipid films were examined with electron microscopy and image analysis. The observations that the molecular packing for the two Ig is very different and that the IgE lattice "melts" at significantly lower temperatures are consistent with IgE being less flexible (Uzgiris, 1987). X-ray crystal structures are available for Fab and IgG-Fc fragments (Saul et al., 1978; Deisenhofer, 1981) and for hinge-deleted mutant human IgG1 (Rajan et al., 1983; Silverton et al., 1977). The hinge deletions in the latter case were probably critical for crystallization due to the elimination of flexibility. Interestingly, hinge-deleted IgG have little or no effector activity.

Several mutually consistent resonance energy transfer distance measurements carried out in our laboratory have shown that IgE bound to FcεRI on RBL cell membranes has a bent structure. In particular, the average distance measured between the carboxy (C)-terminal end and the two amino (N)-terminal ends is substantially less than the value expected for a planar Y- or T-shape (Zheng et al., 1991). Roughly the same average end-to-end distance was obtained for IgE in solution. The contribution of segmental flexibility to that average could not be assessed in those experiments. Prior to the present study, similar energy transfer measurements had not been carried out for IgG, either in solution or bound to membrane receptors. Results from sedimentation and small-angle X-ray scattering experiments on IgG and IgE in solution indicate some static or dynamic bending in these structures (Burton, 1990).

The experiments described here employed energy transfer to compare directly the conformations of IgG1 and IgE in solution and bound to their respective membrane receptors. For these purposes we employed genetically engineered derivatives of human IgG1 (γ/Cγ3*) and murine IgE (ε/Cγ3*) which have identical antigen binding sites specific for DNS. They also have identical C-terminal domains (Cγ3) into which a cysteine residue has been introduced near the C-terminus, allowing a fluorescent probe to be placed at this position. With these derivatives we could measure the energy-transfer distance between C-terminal and N-terminal ends and observe how the distance varies in the presence of agents that might alter structural conformation or flexibility. We also employed the collisional quenching energy-transfer method of Gryczynski et al. (1988) which provides information about the distribution of distances comprising the average value obtained with standard energy-transfer measurements. With the latter approach the contribution of flexibility to the conformations of IgE and IgG1 was assessed.

EXPERIMENTAL PROCEDURES

Chemicals. Fluorescein maleimide (FM) was obtained from Molecular Probes, Inc. (Eugene, OR). Dithiothreitol (DTT), N^{ϵ}-[[5-(dimethylamino)naphthalen-1-yl]sulfonyl]-L-lysine (DNS-Lys), pepsin, polyclonal human IgG, and Sephadex G-50 were purchased from Sigma Chemical Co. (St. Louis, MO). Eosin–DNS (ED) was a gift from Drs. L. Lee and V. Oi (Becton Dickinson Monoclonal Center, Mountain View, CA) and was prepared and purified as described previously (Zheng et al., 1991). Rat anti-mouse Cε3 antibodies (23G3 and R1E4; Keegan et al., 1991) were gifts from Dr. D. Conrad (Medical College of Virginia) and Dr. M. Kehry (DNAX, Palo Alto, CA). Mouse anti-human antibodies specific for Cγ2 and Cγ3 (JD312, F10F, A55, NL16, VC9, WC2; Nik Jaafar et al., 1983) were gifts from Dr. R. Jefferis (University of Birmingham, U.K.).

IgE and IgG1 Preparations. The monoclonal antibodies, human IgG1 (Dangl et al., 1988) and mouse IgE (27–74; Oi et al., 1984), used in this study are derived from a transfectoma and a switch variant cell line, respectively, and have identical antigen binding sites specific for DNS. The IgG1 mutant C444 (designated herein as γ/Cγ3*) was genetically engineered as described (Shopes, 1992) by replacing the codon for serine 444 (Eu notation; Kabat et al., 1987) located near C-terminus of Cγ3 by a codon for cysteine. This point-mutated Cγ3 domain is designated Cγ3*. The chimeric IgE, ε/Cγ3 and ε/Cγ3*, were prepared genetically from these IgE and IgG1 as described (Shopes et al., 1990; Zheng et al., 1991): ε/Cγ3 is the same as IgE except that the exon coding for Cε4 was replaced by the exon coding for Cγ3; ε/Cγ3* is the same as IgE except that the exon coding for Cε4 was replaced by the exon coding for Cγ3*. The recombinant Ig were secreted from transfectomas derived from A5C13 (ε/Cγ3 and ε/Cγ3*) or SP2/0 (γ/Cγ3*) cells and purified by affinity chromatography as previously described (Shopes et al., 1990; Weetall et al., 1990). Protein concentrations were determined by UV absorbance measurements with a Hewlett Packard 8451A UV–visible spectrophotometer [IgE: $\epsilon_{280} = 1.62$ mL/(mg·cm), $M_r = 184\,000$; IgG1: $\epsilon_{280} = 1.4$ mL/(mg·cm), $M_r = 150\,000$]. Protein compositions were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE), with or without the addition of 20 mM DTT to reduce disulfide bonds, using a Bio-Rad Mini-Protean II system and standard procedures (Holowka & Baird, 1983b).

Modification and Enzymatic Digestion of Ig. C-Terminal sulfhydryl groups within the Cγ3* domain of ε/Cγ3* were selectively labeled by FM as described previously (Zheng et al., 1991). Selective modification of the Cγ3* sulfhydryls within γ/Cγ3* was optimized from this procedure. In brief, γ/Cγ3* was partially reduced in PBS (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM EDTA) containing 50 μM DTT for 40 min at room temperature before addition of FM in methanol stock solution to a final concentration of 60 μM and further incubation for 10 min. Free FM was removed by centrifugation through a Sephadex G-50 gel filtration column (Penefsky, 1977). These labeling procedures yielded the products designated FM-ε/Cγ3* and FM-γ/Cγ3*. The respective control samples are FM-ε/Cγ3 and FM-IgG1 which were prepared by parallel modification reactions. The stoichiometry of FM labeling was determined from UV absorbance measurements as described previously (Zheng et al., 1991).

FM-γ/Cγ3* and FM-IgG1 at 0.3 mg/mL were digested with pepsin (1% w/w) in 0.2 M sodium acetate buffer (pH 4.5) at 37 °C as described (Stanworth & Turner, 1986). The reaction was quenched by addition of solid Trizma base to raise the pH to 8.0 followed by cooling to 4 °C. Digestion products were analyzed visually after SDS–PAGE with UV light to detect fluorescence and with Coomassie blue stain to detect protein as previously described (Zheng et al., 1991). In some cases relative fluorescence in individual bands in the gel was determined by band excision and spectroscopic measurement of the extracted fluorescence (Holowka & Baird, 1983b).

Preparation of Plasma Membrane Vesicles. Vesicles containing FcεRI receptors for IgE were prepared from RBL-2H3 cells (Taurog et al., 1979) by chemically inducing blebs to form and pinch off from adherent cells as described (Baird & Holowka, 1985). They were used in energy-transfer measurements after extensive dialysis against PBS containing 0.02 unit/mL aprotinin. Receptors on vesicles were saturated

by incubation with 3–5-fold excess IgE (or variant) in PBS at room temperature for 2 h, followed by 10-fold dilution in PBS, centrifugation at 25000*g* for 40 min, and resuspension of the pelleted vesicles in PBS. Final suspensions of vesicles contained about (5–7) × 10^6 cell equivalents of vesicles/mL as determined by the relative binding of ^{125}I-IgE (Holowka & Baird, 1983a).

The nitrogen cavitation method (Ferber et al., 1972) was used to prepare vesicles from U937 cells (American Tissue Type Culture, Bethesda, MD; CRL 1593) which have ~2 × 10^4 FcγRI receptors/cell (Shopes et al., 1990). Briefly, 5–7 mL containing 4 × 10^7 cells/mL was suspended in a buffer containing 10 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 140 mM NaCl, 50 μg/mL deoxyribonuclease I, 0.5 mM phenylmethanesulfonyl fluoride, and 0.02 unit/mL aprotinin. The cell suspension was maintained at 4 °C with constant stirring and subjected to a pressure of 500 psi for 15 min in a nitrogen cavitation bomb (Artisan Industries, Waltham, MA). The suspension was released dropwise to atmospheric pressure and then centrifuged at 1000 rpm for 10 min in a Sorvall GLC-2B centrifuge. The supernatant containing the plasma membrane vesicles [(2–4) × 10^7 cell equivalents/mL] was divided into three identical samples. These samples were then treated to saturate the FcγRI receptors with one form of IgG1. One aliquot was preincubated for 2 h with unlabeled polyclonal human IgG (final concentration 0.1 mg/mL) to block receptors and provide a specificity control. Then FM-γ/Cγ3* was added (final concentration 3 μg/mL) to that sample and to a second aliquot (containing no unlabeled IgG), and the incubation was continued for ~3 h. FM-IgG1 (final concentration 3 μg/mL) was added to the third aliquot before the ~3-h incubation period. The samples were centrifuged at 25000*g* for 40 min, and the membrane pellets were resuspended in PBS as described above for IgE and the RBL cell vesicles.

Solubilized FcγRI Receptors. The three U937 vesicle samples with FcγRI receptors saturated with one form of IgG1 were prepared as described above. The membrane were then solubilized by incubation with 10 mM CHAPS for 10 min at 4 °C. Before fluorescence measurements, the samples were centrifuged at 30 000 rpm for 20 min to remove insoluble material.

Fluorescence Measurements. Samples (2 mL) maintained at 22 °C were stirred continuously in 10 × 10 × 48-mm acrylic cuvettes (Sarstedt Co.) in an SLM 8000 fluorimeter operated in ratio mode. The fluorescein fluorescence of FM was monitored with optimal excitation and emission wavelengths of 480 and 510 nm, respectively. The shape of the fluorescein emission spectrum was found to be the same for all FM-labeled samples. Therefore, the fluorescence observed at the optimal wavelengths for each sample was assumed to be proportional to its quantum yield (Q), after correction for background contributions that were assessed in parallel samples. The steady-state anisotropy was measured as previously described (Holowka & Baird, 1983a).

For the energy-transfer experiments the critical distance R_0 was calculated from (Forster, 1959)

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_d n^{-4})^{1/6}\ \text{Å} \qquad (1)$$

The overlap integral (J) was calculated as previously described (Cantley & Hammes, 1975); the orientation factor (κ^2) was taken as 2/3 (Holowka & Baird, 1983b); the refractive index of the medium (n) was taken as 1.4. The quantum yield (Q_d) for fluorescein in the FM-ε/Cγ3* and FM-γ/Cγ3* was determined with sodium fluorescein in 0.1 N NaOH (Q_d = 0.91; Weber & Teale, 1957) as a standard.

The intramolecular energy-transfer distance, R, between the FM (donor) located at the C-termini of FM-γ/Cγ3* and FM-ε/Cγ3* and the ED (acceptor) added to the antigen binding sites at the N-termini, was determined for these Ig in solution (~6–10 nM) and bound to receptors on the vesicles (~2–6 nM) or solubilized receptors (~2 nM). An equivalent amount of the respective vesicle preparation (but with vesicle receptors saturated with unlabeled Ig) was added to samples for the measurements of FM-labeled Ig derivatives in solution. As described previously, this addition eliminates nonspecific association of ED with the FM-labeled Ig and nonspecific adsorption of the FM-labeled Ig to the cuvette walls (Zheng et al., 1991).

The energy-transfer experiments followed the same basic procedure described previously (Zheng et al., 1991). The fluorescein fluorescence of FM-γ/Cγ3* (or FM-ε/Cγ3*) was monitored as ED was added in microliter aliquots to saturate the binding sites (Q_{da} at saturation) and then as excess DNS-Lys was added subsequently to displace the ED (Q_d at completion). These fluorescence values were corrected by subtracting the corresponding signals from the background control sample FM-IgG1 (or FM-ε/Cγ3) which was monitored in parallel. The acceptors ED were assumed to occupy the two binding sites at equal distances (R) from the donor FM, and R was calculated from efficiency of energy transfer E as follows:[2]

$$E = 1 - Q_{da}/Q_d = 2(R_0/R)^6/[1 + 2(R_0/R)^6] \qquad (2)$$

Analysis of Distance Distributions. The distributions of distances (r) between C-terminal FM and N-terminal ED for FM-ε/Cγ3* and FM-γ/Cγ3* were generated from steady-state energy-transfer measurements according to the method of Gryczynski et al. (1988). The method depends upon systematic changes in the Q_d (and, consequently, R_0; eq 1) caused by the addition of varying amounts of a collisional quencher. I^- was used as the quenching species, and NaI was added continuously (360 nmol/s) from a stock solution (4.2 M) to the stirred sample with a Harvard microliter syringe pump. The FM fluorescence was monitored as a function of [I^-] and corrected for dilution. In the absence of I^- the fluorescent samples provide values of Q_d, Q_{da}, and R_0. In the presence of I^- these values become Q_d^q, Q_{da}^q, and R_0^q, respectively, and they decrease with increasing [I^-].

The distribution of distances was assumed to be described by a symmetrical Gaussian function

$$P(r) = (\sigma\sqrt{2\pi})^{-1} \exp[-0.5((r - \rho)/\sigma)^2] \qquad (3)$$

where ρ is the average (and most probable) distance and σ is the standard deviation of the distribution. The half-width of the distribution (full width at half-maximum is given by hw = 2.354σ. Theoretical efficiencies of energy transfer corresponding to possible combinations of the σ and ρ parameters

[2] It is possible that the structural conformations available to IgE or IgG1 place the C-terminal end closer to one N-terminal end (e.g., coplanar sideways bending of a Y shape). This would not make a large difference in the distance extracted from our energy-transfer measurements. For example, if R (or r) corresponds to two acceptors being equally spaced from the donor and R' (or r') corresponds to only one acceptor being close enough to the donor for detectable energy transfer, then $R' = R \times 2^{1/6} = (0.89)R$ in eq 2, and $r' = (0.89)r$ in eq 4. Other types of asymmetries would have similar effects. In general, each distance r_i contributes to the observed energy-transfer efficiency as r_i^{-6}, i.e., $E = (1/n)\sum a_i(R_0/r_i)^6/[1 + (1/n)\sum a_i(R_0/r_i)^6]$, where a_i is a weighting factor and $\sum a_i = n$.

were calculated according to[3]

$$E_c^q = \int_{10\text{Å}}^{\infty} P(r)2(R_0^q)^6/[2(R_0^q)^6 + r^6]\, dr \quad (4)$$

This calculation is not sensitive to the lower limit of integration in the range r = 0–10 Å because $P(r) \sim 0$ in this range (eq 3). As illustrated below, these theoretical values were compared to the efficiencies measured experimentally as a function of R_0^q. The best fitting combination of ρ and σ yielded the minimum value of χ^2:

$$\chi^2 = (1/\nu)\sum[(E^q - E_c^q)/\Delta E]^2 \quad (5)$$

where ΔE is the estimated error in the measured efficiencies and ν is the number of points used in the analysis.

This method was applied to determine distance distributions for IgE in solution and bound to receptors on vesicles (with FM-ϵ/Cγ3*) and for IgG1 in solution (with FM-γ/Cγ3*). As an illustrative example, consider the case of IgE in solution and the data shown in Figure 3A. The FM fluorescence was monitored as a function of [I^-] for three samples: (1) FM-ϵ/Cγ3* in the presence of a saturating amount of ED and excess DNS-Lys (donor), (2) FM-ϵ/Cγ3* in the presence of saturating ED (donor–acceptor), and (3) FM-ϵ/Cγ3 in the presence of saturating ED and excess DNS-Lys (background). Curve 3 was subtracted from curves 1 and 2 to yield the curves shown in Figure 3B; the top curve represents Q_d^q and the bottom curve represents Q_{da}^q as functions of [I^-]. For each sample [I^-] = 0 corresponds to the nonquenched fluorescence (Q_d and Q_{da}). The data points were smoothed by fitting with a 5th-order polynomial function as shown by the solid lines. Points corresponding to 20 values of [I^-] spaced evenly across the concentration range were selected from these fitted curves for the following analysis. For each of these [I^-], the following experimental values were calculated:

$$E^q = 1 - Q_{da}^q/Q_d^q \quad (6)$$

$$R_0^q = R_0(Q_d^q/Q_d)^{1/6} \quad (7)$$

These experimental E^q (eq 6) were compared to theoretical E_c^q (eqs 3 and 4) using an iterative procedure and eq 5 to determine the best values of ρ and σ. In the first step $\rho = R$ from Table I was chosen as the initial estimate, and then various values of σ were tested to find the minimum χ^2. The σ corresponding to this minimum was then held constant as ρ was varied to find the minimum χ^2. This procedure was continued until the values of ρ and σ converged to their best values. The plots in Figures 4 and 5 show how this analysis yielding the minimal χ^2 clearly provides best fit values for ρ and σ (hw). In order to confirm that a global minimum for (ρ, σ) was obtained, values covering a reasonable range of possible ρ (e.g., 70–140 Å for IgE) were also tested as initial estimates.

[3] The limiting anisotropy value for fluorescein (480-nm excitation) is 0.4 in basic glycerol solution (Chen & Bowman, 1965). In the case of membrane-bound FM-ϵ/Cγ3*, the difference between the observed value for A of 0.21 (Table I) and the limiting value of 0.4 must be due to probe rotation and/or segmental motion; the global rotation of the IgE–receptor complex within the membrane (or the tumbling motion of the membrane vesicles) is too slow to contribute to the depolarization observed (Holowka et al., 1990). It is difficult to estimate how much the Ig tumbling motion contributes to the measured anisotropies for FM-γ/Cγ3* and FM-ϵ/Cγ3* in solution, but it is likely that the probe rotation and internal flexibility occur at least as much for these samples as for FM-ϵ/Cγ3* bound to membranes.

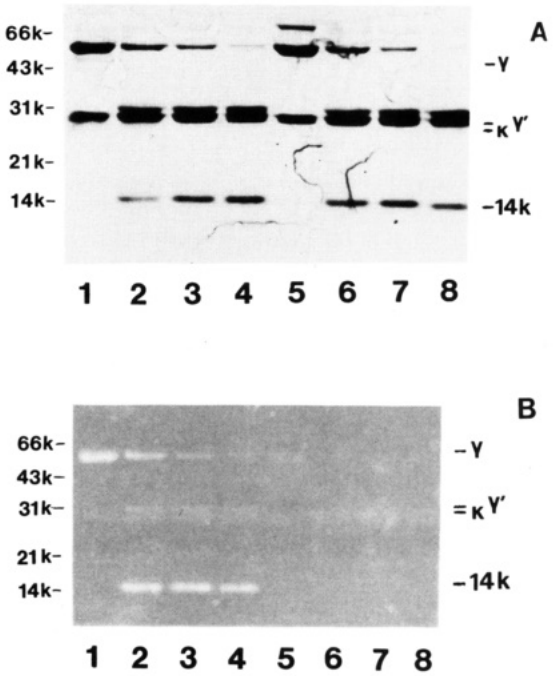

FIGURE 1: SDS–PAGE analysis of IgG1 derivatives. Coomassie blue stain (A) and fluorescence (B) are shown for the same gel (12.5% acrylamide) run under reducing conditions. Equal amounts (~8 μg) of FM-γ/Cγ3* (lanes 1–4) and FM-IgG1 (lanes 5–8) were loaded on to the gel after pepsin digestion for 0 h (lanes 1 and 5), 4 h (lanes 2 and 6), 10 h (lanes 3 and 7), and 24 h (lanes 4 and 8). Number labels indicate the apparent molecular mass, and γ, γ', and κ indicate the position of IgG heavy chain, clipped heavy chain, and light chain, respectively.

RESULTS

Fluorescence Modification of γ/Cγ3 and Energy Transfer between the C-Terminal and N-Terminal Ends.* SDS–PAGE analysis of purified γ/Cγ3* in the absence of reducing agents shows 50–70% of the protein migrating as monomeric Ig ($\gamma_2\kappa_2$; γ and κ denote the Ig heavy and light chain, respectively). The remainder migrates with apparent molecular mass corresponding to dimers [$(\gamma_2\kappa_2)_2$] (data not shown). This covalent dimerization probably results from intermolecular disulfide bond formation between the cysteines introduced into the Cγ3 domains (Shopes, 1992). In the absence of reducing reagents very little FM is incorporated into γ/Cγ3*. After reduction of disulfide bonds with >100 μM DTT, FM becomes incorporated into both γ heavy and κ light chains as visualized on SDS–PAGE gels with UV illumination (data not shown). Selective modification of the Cγ3 sulfhydryls to prepare FM-γ/Cγ3* is achieved after reduction with 50 μM DTT. Although the resulting stoichiometry is low (0.3 mol of FM/mol of γ/Cγ3* as determined by absorption measurements), IgG1 subjected to identical reaction conditions shows substantially less FM incorporation (<0.03 mol of FM/mol of γ/Cγ3*).

SDS–PAGE analysis of FM-γ/Cγ3* without further reduction shows >90% of the protein as monomer and only traces of $(\gamma_2\kappa_2)_2$ and $\gamma\kappa$ at positions corresponding to higher and lower molecular masses, respectively (data not shown). The 10-fold difference in labeling stoichiometry between FM-γ/Cγ3* and FM-IgG1 was confirmed by quantifying the fluorescence in the monomer bands excised from the gel lanes of these two samples (data not shown). SDS–PAGE of FM-γ/Cγ3* in the presence of reducing agents shows that 90% of the FM fluorescence is associated with the heavy chain, whereas <10% fluorescence is detected with the light chain (Figure 1, lane 1). Very little fluorescence is detected with

Table I: Energy-Transfer Parameters for Fluorescently Labeled Ig

sample[a]	A_d[b]	E[c]	R (Å)[c]	ρ (Å)[d]	hw (Å)[d]	R_p[h]
IgE	0.14	0.20	71	75	24	71.8
IgE-FcεRI(mem)[e]	0.21	0.22	69	71	12	70.0
IgG1	0.12	0.15	75	100	89	76.8
IgG1-FcγRI(mem)[e]	nd[g]	≤0.08	>85	nd	nd	nd
IgG1-FcγRI(sol)[f]	nd	≤0.05	>92	nd	nd	nd

[a] Experimental samples used in these measurements were FM-ε/Cγ3* (FM-ε/Cγ3 as control) for IgE and FM-γ/Cγ3* (FM-IgG1 as control) for IgG1. In the energy-transfer experiments fluorescein (as FM) was the donor probe and eosin (as ED) was the acceptor probe. For FM-ε/Cγ3*, Q_d = 0.28 (Zheng et al., 1991); for FM-γ/Cγ3*, Q_d = 0.31 (data not shown). For the fluorescein–eosin energy-transfer pair, R_0 = 50 Å (Zheng et al., 1991). [b] Steady state anisotropy (A_d) for fluorescein on FM-Ig derivative at 23 °C; steady-state anistropy for ED in antigen binding site is 0.28 (Zheng et al., 1991). [c] Values for IgE taken from energy-transfer experiments of Zheng et al. (1991); values for IgG1 are from repetitions of experiments shown in Figure 2. E is the efficiency of energy transfer; R is the average distance between donors and acceptors calculated from E and eq 2. The values shown are averages (or limits) from four separate experiments with each type of sample; the only exception is IgG1-FcγRI(sol) for which only two experiments were done. The standard deviations from the R averages shown are as follows: IgE, 0.9; IgE-FcεRI(mem), 1.2; IgG1, 0.8. [d] Values taken from experiments and theoretical calculations depicted in Figures 3–6 in which energy transfer varies as a function of [I⁻]. ρ is the average of an assumed Gaussian distribution of distances between donors and acceptors; hw is the full width at half-maximum of this distribution. [e] Ig samples were bound to receptors on plasma membrane vesicles: IgG1 samples were bound to FcγRI on vesicles derived from U937 cells; IgE samples were bound to FcεRI on vesicles derived from RBL cells. [f] IgG1 samples were bound to FcγRI receptors solubilized from U937 cell membranes. [g] Not determined. [h] Weighted average R values calculated from the distribution of end-to-end distances r based on varied R_0 (Figures 3–6) for comparison with values from energy-transfer measurements based on single R_0 (Figure 2). Thirty points symmetrically located across each curve in Figure 6 were taken. The E_r corresponding to these r were calculated from eq 2, and these were weighted by the corresponding normalized $P(r)$ and summed. Then R_p was calculated from this weighted, summed E_r according to eq 2.

either band for FM-IgG1 (Figure 1, lane 5). The specificity of the FM reaction with the Cγ3* sulfhydryls is demonstrated with pepsin digestion which was shown previously to produce a fragment corresponding to $(Fab')_2$ and pFc' (essentially Cγ3) from human IgG1 (Stanworth & Turner, 1986). On SDS–PAGE gels (Figure 1) pepsin digestion of FM-γ/Cγ3* results in the generation of 14-kDa band corresponding to Cγ3* that contains 80% of the total fluorescence. Bands corresponding to the γ' heavy and κ light chain components of the $(Fab')_2$ fragment contain very little fluorescence. After similar pepsin digestion of FM-IgG1, no fluorescence is detected in the 14-kDa band. The low amount of fluorescence found in the bands arising from the $(Fab')_2$ fragment for FM-IgG1 is similar to that for FM-γ/Cγ3*. These results show that FM-IgG1 is a good control for FM-γ/Cγ3* in energy-transfer experiments.

With FM-γ/Cγ3* we were able to examine a structural dimension of IgG1 by measuring energy transfer between fluorescein (FM) conjugated to sulfhydryl groups at the C-terminus and eosin in the form of ED located in the DNS-specific binding sites at the two N-termini. We carried out similar experiments previously for IgE with FM-ε/Cγ3* (Zheng et al., 1991). Table I shows fluorescence and energy-transfer parameters for FM-γ/Cγ3* and FM-ε/Cγ3* in solution and for these Ig bound to their receptors. The steady-state anisotropy for the donor FM in FM-γ/Cγ3* is similar to that for FM in FM-ε/Cγ3*. These values are less than would be expected for fluorescein probes that are rigidly attached.[3] A low anisotropy value of 0.28 is also observed for ED in the antigen binding site (Zheng et al., 1991). These

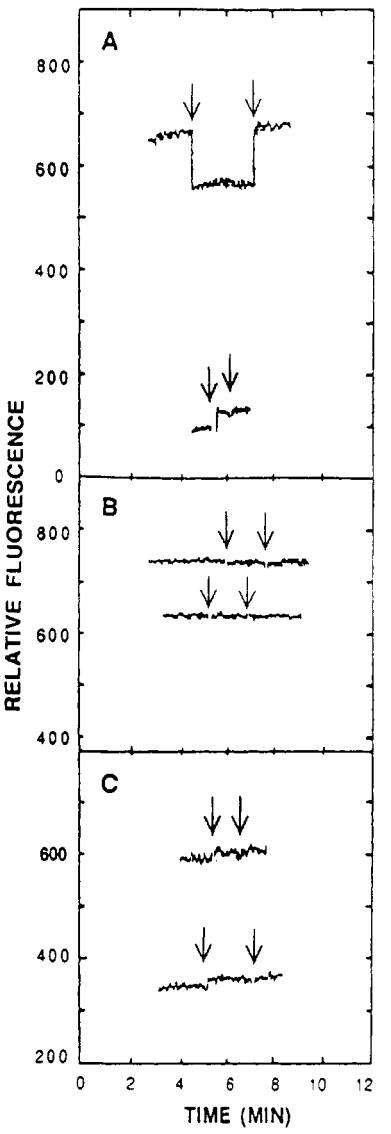

FIGURE 2: Measurements of energy transfer between donors FM at the Fc C-terminus and acceptors ED in the Fab N-terminal antigen binding sites, for FM-γ/Cγ3* in solution (A), FM-γ/Cγ3* bound to receptors on membrane vesicles (B), and FM-γ/Cγ3* bound to solubilized receptors (C). Fluorescein fluorescence was monitored as a function of time. The top trace in each panel represents the signal of the FM-γ/Cγ3* sample, and the lower trace is the signal of the control sample FM-IgG1. Arrows in each trace indicate the addition of ED (final concentration 40 nM) and then the subsequent addition of a large excess of DNS-Lys (final concentration ≥2 μM) to displace the antibody-bound ED.

data indicate some freedom of movement of the donor and acceptor probes during the donor excited-state lifetime, which supports our approximation of $\kappa^2 = 2/3$ in the calculation of the R_0 for the energy-transfer experiments (eq 1).

Energy transfer was measured for FM-γ/Cγ3* in solution as shown by the representative trace in Figure 2A (upper trace). Addition of a saturating amount of ED to FM-γ/Cγ3* causes immediate quenching of the fluorescein fluorescence. Because eosin fluorescence contributes a small amount to the signal at the monitoring wavelength, this quenched fluorescence value (Q_{da}) is compared to the fluorescence after the subsequent addition of an excess of DNS-Lys which effectively competes with ED for the DNS binding sites but does not quench the fluorescence (Q_d). A similar measurement for the control sample of ED and FM-IgG1 shows no energy transfer and no change in fluorescence due to the addition of DNS-Lys (Figure 2A, lower trace).

After subtraction of the fluorescence of the control sample the efficiency of energy transfer between the probes at the C-terminal and N-terminal ends was determined to be 0.15 (average value from four separate experiments). Assuming two acceptor probes equidistant from a single donor probe, this corresponds to a distance of 75 Å (eq 2; Table I). This end-to-end distance is shorter than expected for a planar T-shaped or Y-shaped IgG1[4] and suggests an average conformation where the Fc segment is asymmetrically displaced relative to the Fab segments, possibly due to flexibility.

The same energy-transfer measurement was made for FM-γ/Cγ3* bound to FcγRI receptors on plasma membrane vesicles derived from U937 cells (Figure 2B) and for FM-γ/Cγ3* bound to FcγRI receptors solubilized from these vesicles (Figure 2C). The number of FcγRI receptors per U937 cell is relatively low, and correspondingly, the fluorescence signal-to-noise ratio for the vesicle sample is low. Little if any energy transfer is observed in the presence of ED. From measurements on four separate vesicle samples we determined $E \leq 0.08$ corresponding to a distance $R \geq 85$ Å. The solubilized FM-γ/Cγ3*-FcγRI samples had a better signal-to-noise ratio (Figure 2C), and we determined $E \leq 0.05$, corresponding to $R > 92$ Å from two separate experiments. These values are included in Table I. Control samples testing for nonspecific binding to the U937 cell receptors had receptors blocked by polyclonal IgG before addition of FM-γ/Cγ3* and washing; the traces show very little fluorescence and no energy transfer (data not shown).

End-to-End Energy Transfer for FM-ϵ/Cγ3 and FM-γ/Cγ3* in the Presence of Anti-Ig Domain Antibodies.* The energy-transfer distances obtained for both FM-ϵ/Cγ3* (Zheng et al., 1991; Table I) and FM-γ/Cγ3* (Table I) suggest asymmetric structures, on average, for IgE and IgG1,[4] but further experiments are required to distinguish between rigid bent structures and very flexible structures. In one approach we employed monoclonal antibodies specific for particular Ig domains in an attempt to alter the flexibility in a manner that could be detected by energy transfer between the probes at the N- and C-terminal ends. The results from these experiments are presented in Table II. Antibodies 23G3 and R1E4 are both specific for Cϵ3; 23G3 does not prevent IgE from binding to FcϵRI on cells, whereas R1E4 is a potent inhibitor and is thought to bind at or near the site of receptor binding (Keegan et al., 1991). Saturating amounts of 23G3 cause a 25% reduction in the efficiency of energy transfer (corresponding to an increase in apparent distance of about 3 Å) for FM-ϵ/Cγ3*, and saturating amounts of R1E4 cause a 15% increase (apparent distance decreased by about 2 Å). When 23G3 and R1E4 are added together, an additive effect is observed, resulting in reduction in E of 15% (Table II). Neither of these antibodies cause any significant effect on the efficiency of energy transfer when they are added in saturating amounts to samples containing FM-ϵ/Cγ3* bound to receptors on membrane vesicles (data not shown).

Antibodies specific for FM-γ/Cγ3* epitopes within Cγ2 (JD312, F10F, A55, and NL16) and within the Cγ2 and Cγ3 interface (VC9 and WC2) Nik Jaafar et al., 1983) were also tested (Table II). These antibodies all cause a reduction (27–67%) in the efficiency of energy transfer (apparent distance increase of 5–17 Å), consistent with the possibility that they are reducing the range of segmental motion within FM-γ/Cγ3*. Taken together, the results shown in Table II clearly indicate that binding of anti-Ig antibodies can perturb the structure of IgE and IgG1 such that the average distance between N-terminal and C-terminal ends is altered. Both Ig appear to have some flexibility, with IgG1 possibly more flexible than IgE, but the absolute degree of flexibility is not readily assessed by this approach.

Table II: Efficiency of Energy Transfer Between C-Terminal and N-Terminal Ends of Ig in the Presence of Anti-Ig Antibodies[a]

sample	antibody (specificity)	E[b]	% change
IgE		0.20	
IgE	23G3 (anti-Cϵ3)	0.15	−25
IgE	R1E4 (anti-Cϵ3)	0.23	+15
IgE	23G3 + R1E4	0.17	−15
IgG1		0.15	
IgG1	JD312 (anti-Cγ3)	0.08	−47
IgG1	F10F (anti-Cγ3)	0.05	−67
IgG1	A55 (anti-Cγ3)	0.10	−33
IgG1	NL16 (anti-Cγ3)	0.11	−27
IgG1	VC9 (anti-Cγ2,3)	0.08	−47
IgG1	WC2 (anti-Cγ2,3)	0.09	−40

[a] Energy-transfer experiments were carried out as described for Figure 2 with the following modification. After a saturating amount of ED was added to the FM-ϵ/Cγ3* (for IgE) or FM-γ/Cγ3* (for IgG1), the sample was titrated with the indicated antibody until a maximal change in the fluorescence signal occurred. Then DNS-Lys was added as in the standard experiment. The control samples (FM-ϵ/Cγ3 for IgE and FM-IgG1 for IgG1) were treated in the same manner for corrections in parallel measurements. [b] Average energy-transfer efficiencies from two independent measurements for each sample. Deviations from these averages are <0.02.

Table III: Efficiency of Energy Transfer between C-Terminal and N-Terminal Ends of Ig in the Presence of Guanidine Hydrochloride[a]

treatment	IgE	IgG1
PBS buffer	0.20	0.15
3 M guanidine hydrochloride[b]	≤0.03	0.11
dialysis into PBS after guanidine hydrochloride[c]	0.19	0.15

[a] Energy-transfer experiments were carried out as described for Figure 2 with the indicated alteration in sample treatment. The labeled Ig derivatives used for IgE and IgG1 are the same as described in Table I (footnote *a*). Values in the first row are the average of 4 experiments (SD = 0.01); values in the second and third rows are the average of 3 experiments (SD ≤ 0.02). [b] Samples were incubated in PBS containing 3 M guanidine hydrochloride for 0.5 h at 22 °C before the addition of ED in the energy-transfer measurement. [c] After energy-transfer measurements were made on samples in 3 M guanidine hydrochloride (row 2), they were extensively dialyzed at 4 °C in PBS buffer in the presence of ≥0.1 μM ED and the energy-transfer measurements were repeated.

End-to-End Energy Transfer in the Presence of a Mild Denaturant. We employed 3 M guanidine hydrochloride to explore the possibility that IgE and/or IgG1 has a rigid bent structure that is maintained by noncovalent interactions between amino acid residues. These interactions would be reduced in the presence of the denaturant, and we would expect the end-to-end distance to increase as the Ig becomes more elongated. We found that DNS ligands remain specifically bound to FM-ϵ/Cγ3* and FM-γ/Cγ3* in the presence of up to 4 M guanidine hydrochloride for 24 h as reported previously for other anti-DNS Ig (Cathou & Werner, 1970). As shown in Table III, 3M guanidine hydrochloride causes a dramatic decrease in the efficiency of C-terminus to N-terminus energy transfer for FM-ϵ/Cγ3* such that the apparent distance goes from 71 Å to ≥100 Å. For FM-γ/Cγ3* this treatment causes a much smaller change in the energy transfer corresponding to an apparent distance change from 75 to 80 Å. The energy

[4] If we assume each Ig domain is 40 Å long (Amsel & Poljak, 1979), then we estimate the end-to-end length for a planar extended IgE to be in the range of 140–200 Å, corresponding to angles ranging from 180° to 0° between the major axes of the two Fab segments. For IgG1 we estimate this end-to-end length to be in the range of 120–170 Å, assuming the hinge segment is 10 Å long (Dangl et al., 1988).

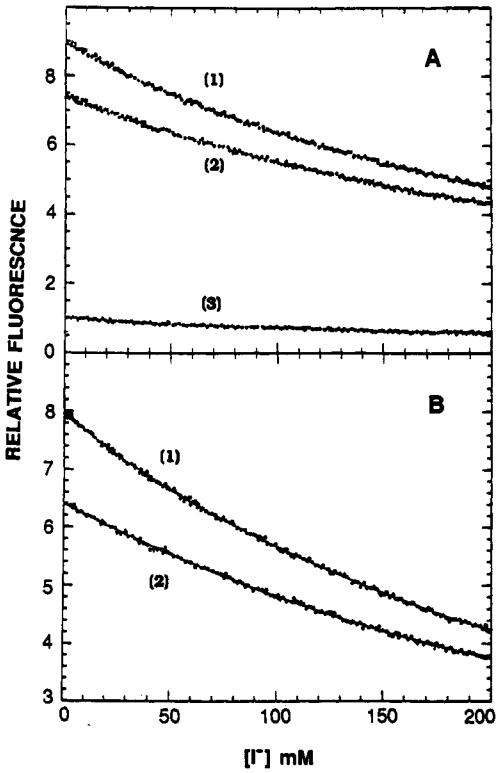

FIGURE 3: Fluorescein quenching of FM-ϵ/Cγ3* in solution as a function of quencher I^- concentration in the absence (1) or presence (2) of acceptors ED. (A) Raw data including the background signal represented by the FM-ϵ/Cγ3 sample (3). (B) Data corrected by subtraction of the background signal and fitted with a polynomial equation to provide a smooth curve.

transfer efficiencies were restored to nearly their original values after removal of the guanidine hydrochloride by dialysis. This indicates that the unfolding caused by this denaturant is largely reversible.[5] The large difference in effects caused by guanidine hydrochloride for FM-ϵ/Cγ3* compared to FM-γ/Cγ3* suggests that noncovalent interactions within the polypeptide chains that stabilize a bent structure are more likely for IgE than for IgG1.

Systematic Alteration of R_0 To Determine the End-to-End Distance Distribution. We employed I^- as a collisional quencher to alter the quantum yield for the fluorescein donor probe and thereby the R_0 for the fluorescein–eosin energy-transfer pair. Stern–Volmer plots confirmed that the fluorescence quenching provided by I^- is collisional rather than static in nature (Gryczynski, 1988). For example, for FM-ϵ/Cγ3* a plot of Q_d/Q_d^q vs $[I^-]$ is linear for $[I^-] = 0$–200 mM (slope = 4.4×10^{-3} mM^{-1}) at 22 °C and linear over this concentration range with a steeper slope (6.3×10^{-3} mM^{-1}) at 37 °C (data not shown). Plots of $(Q_d/Q_d^q - 1)/[I^-]$ vs $[I^-]$ are also linear with similar slopes. As described by Gryczynski et al. (1988), energy transfer measured as a function of varying R_0 can be used to recover a theoretical distribution of end-to-end distances corresponding to the conformations available to the structure. This method assumes a Gaussian shape and provides the average distance (ρ) as well as the half-width (hw) of the distribution. In this manner the flexibility of FM-ϵ/Cγ3* and FM-γ/Cγ3* could be directly assessed.

Figure 3A shows the data from an experiment of this type

[5] It appears that the 3 M guanidine hydrochloride treatment causes some structural perturbation in the receptor binding region of IgE that is not reversed by dialysis. In particular, we found that high-affinity binding to RBL cells is lost after this treatment.

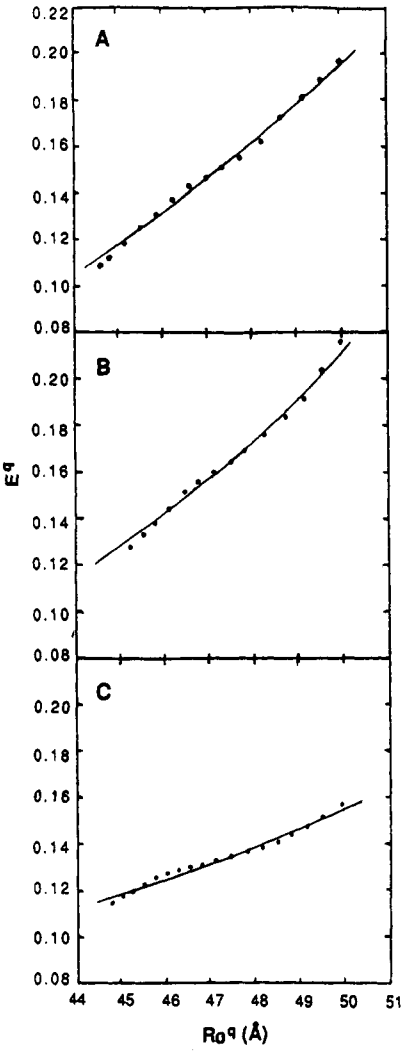

FIGURE 4: Least-squares fits of the end-to-end energy-transfer efficiencies (E^q) measured as a function of R_0^q. The R_0^q and E^q were calculated with eqs 6 and 7 using data from experiments such as that shown in Figure 3B. The samples together with the parameters providing the best fits (solid lines) with eqs 3–5 are as follows: (A) FM-ϵ/Cγ3* in solution, ρ = 75 Å and hw = 24 Å; (B) FM-ϵ/Cγ3* bound to receptors, ρ = 71 Å and hw = 12 Å; (C) FM-γ/Cγ3* in solution, ρ = 100 Å and hw = 88 Å.

for FM-ϵ/Cγ3*. The fluorescence for FM-ϵ/Cγ3* was measured as a function of $[I^-]$ for samples with saturating amount of the ligand ED and with (donor) or without (donor–acceptor) an excess of DNS-Lys to displace the ED. The control sample FM-ϵ/Cγ3 was measured in parallel. For comparison to the theoretical Gaussian distributions the data were corrected by subtracting the background FM-ϵ/Cγ3 signal and then smoothed with a polynomial function (Figure 3B). Corresponding curves were obtained for FM-ϵ/Cγ3* bound to FcεRI on vesicles (with FM-ϵ/Cγ3 bound to vesicles as a control) and for FM-γ/Cγ3* in solution (with FM-IgG1 as a control) (data not shown).

The points in Figure 4 represent the measured efficiency of energy transfer as a function of R_0 for FM-ϵ/Cγ3* in solution (Figure 4A), for FM-ϵ/Cγ3* bound to FcεRI on vesicles (Figure 4B), and for FM-γ/Cγ3* in solution (Figure 4C). As described in Experimental Procedures, the data were compared to theoretical curves corresponding to different values of ρ and hw, and the solid lines in Figure 4 show how well the data can be fit by the optimal combination. The best fit values for ρ and hw are obtained by χ^2 analyses (eq 5) as shown in Figure 5.

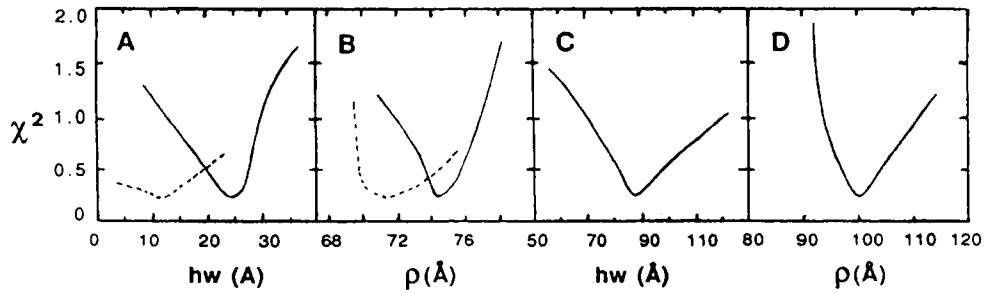

FIGURE 5: Dependence of χ^2 (eq 5) on the values of ρ and hw which parametrize the Gaussian distributions of end-to-end distances for FM-ϵ/Cγ3* in solution (solid lines, A and B), FM-ϵ/Cγ3* bound to receptor (dashed lines, A and B), and FM-γ/Cγ3* in solution (C and D).

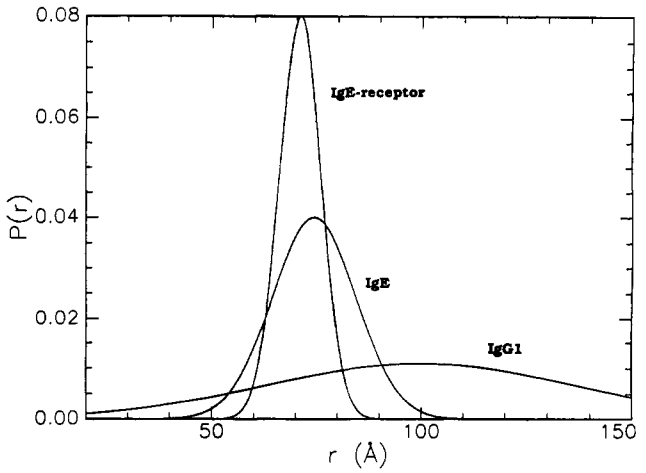

FIGURE 6: Gaussian distributions (eq 3) representing the end-to-end distances determined for IgE in solution (dashed line; ρ = 75 Å, hw = 24 Å), IgE bound to receptor (dotted line; ρ = 71 Å, hw = 12 Å), and IgG1 in solution (solid line; ρ = 100 Å, hw = 88 Å). Experimental samples are as described in Table I, footnote *a*.

Figure 6 shows the Gaussian distributions resulting from these analyses for the three different sets of samples tested. FM-ϵ/Cγ3* bound to FcϵRI on vesicles has the narrowest distribution with ρ = 71 Å, hw = 12 Å. For FM-ϵ/Cγ3* in solution the distribution is somewhat broader and shifted to longer distances, ρ = 75 Å, hw = 24 Å. Similar analysis shows that the end-to-end distance distribution for FM-γ/Cγ3* in solution is distinctly broader with a longer average end-to-end distance: ρ = 100 Å, hw = 88 Å.

DISCUSSION

This study employed resonance energy transfer to compare the structural conformations of murine IgE and human IgG1 in terms of the distance between N- and C-terminal ends. We also assessed the degree to which Ig flexibility and the corresponding distribution of end-to-end distances contributes to the apparent distance value obtained with standard energy-transfer measurements. A direct comparison between isotypes was made possible by the availability of two genetically engineered Ig, γ/Cγ3* and ϵ/Cγ3*, which have identical antigen binding sites for DNS and identical C-terminal domains that were specifically labeled near the C-terminus. These Ig derivatives appear to maintain the structural features of the native Ig: ϵ/Cγ3* binds to FcϵRI receptors on RBL cells as well as native IgE (Weetall et al., 1990; Zheng et al., 1991); γ/Cγ3* binds to FcγRI receptors on U937 cells as well as IgG1 (data not shown), and the monomeric form used here has the same activity in complement assays (Shopes, 1992). For our comparison of end-to-end distances within these Ig, energy transfer was measured between fluorescein (as FM) conjugated to sulfhydryls at the end of the Fc segment and eosin (as ED) located in antigen binding sites at the end of the Fab segments.

This study stemmed from a large number of previous energy-transfer measurements which yielded a consistent picture that IgE bound to FcϵRI receptors has a bent structure such that the axes of the Fab segments and the axis of Fc segment do not form a planar Y- or T-shape (Baird & Holowka, 1988). Confirming this view was our recent direct measurement of the N-terminal end to C-terminal end distance for receptor-bound ϵ/Cγ3*; this was found to be substantially shorter than that expected for a planar structure (Zheng et al., 1991). Unexpectedly, the same measurement made for IgE in solution indicated that this structure is similarly bent. For further evaluation of these results the present study extended the comparison to human IgG1 for which γ/Cγ3* is available. IgG1 has the same basic structure as IgE except that in the place of the Cϵ2 domains (~40 Å long) is a relatively short hinge segment containing about 14 amino acids (~10 Å long) in each of the two γ heavy chains. Consequently, the end-to-end length for IgG1 would be expected to be shorter than that for IgE if both were extended planar molecules. Because of its hinge, IgG1 also would be expected to be more flexible than IgE, as has been supported experimentally (Oi et al., 1984; Dangl et al., 1988).

Our initial energy-transfer efficiency measurements were interpreted with eq 2 in terms of a single apparent (average) end-to-end distance (Table I; R values) based on a single characteristic R_0 for that donor–acceptor pair. The apparent end-to-end distance for IgG1 in solution was determined to be 75 Å, which is substantially less than expected for planar Y-shaped or T-shaped structures,[4] and somewhat longer than the same measurement made previously for IgE in solution (71 Å; Table I; Zheng et al., 1991). For IgG1 bound to FcγRI receptors on membrane vesicles derived from U937 cells or

[6] The uncertainty in κ^2 for FM-ϵ/Cγ3*-FcϵRI is readily estimated with the dynamic depolarization model of Dale and Eisenger (1974) as follows. The dynamic depolarization of a donor or acceptor is calculated as $\langle d' \rangle_d = r_{om}/r_f$, where r_{om} is the anisotropy due to probe rotation that is independent of macromolecule rotation (0.21 for FM on ϵ/Cγ3*-FcϵRI and 0.28 for ED bound to this complex) and r_f is the fundamental or limiting anisotropy (0.4 for fluorescein or eosin; see footnote 3). The calculated values for $\langle d' \rangle_d$ predict half-angles (Ψ) for the probe rotation within a solid cone of 40° and 30° for FM and ED, respectively. Taking the limiting geometries in which donor and acceptor dipoles are confined to cones oriented either parallel or perpendicular to a line joining their centers [Dale and Eisenger, (1974), Figures 12 and 15], the limiting values for κ^2 are determined to be 2.8 and 0.15, respectively, for the parallel and perpendicular orientations. These correspond to respective R_0 values of 64 and 39 Å such that the distance R between FM and ED falls within the range 54 Å < R < 88 Å. Because the probe rotation is not readily assessed for FM-ϵ/Cγ3* and FM-γ/Cγ3* in solution (see footnote 3), the uncertainty in κ^2 cannot be calculated in the same way, but this uncertainty is likely to be similar to or less than that for FM-ϵ/Cγ3*-FcϵRI. Therefore, we conclude that the error in the distances based on the uncertainty in κ^2 is less than 30%.

to these receptors solubilized from the membranes the efficiency of energy transfer is significantly reduced, corresponding to an increase in the apparent end-to-end distance (≥85 Å). In contrast, when IgE binds to FcεRI receptors on vesicles derived from RBL cells, and end-to-end distance decreases slightly (to 69 Å) as compared to the solution form. A plausible scenario for the results obtained is that binding to receptor causes IgG1 to lose flexibility such that its average end-to-end distance is increased and that receptor-bound IgG1 is bent less acutely than receptor-bound IgE or IgE in solution.

Certainty in the values for these average distances is limited by the precision in our measurements of transfer efficiency and also by the accuracy of the distance calculation. The precision of our measurements is indicated by their reproducibility in multiple experiments. As shown in Table I, the standard deviations from four independent experiments are all ≤2% of the distance calculated. The accuracy in the distance calculated depends primarily on uncertainty in the value for κ^2, and this corresponds to an error in the absolute value of R of <30%.[6] The actual value of κ^2 is likely to be similar for all of the IgE and IgG samples compared, and therefore, the differences between the distances measured for these samples are probably real.

We emphasize that the apparent end-to-end distance provided from each of these energy-transfer measurements with a single R_0 represents a weighted average over the distribution of conformations available to these Ig. With this type of measurement it is not possible to distinguish whether the apparent distance determined arises from a rigid structure (single distance) or from a flexible structure (corresponding to some continuum of distances). It is likely that the Ig segmental flexibility will partially determine the apparent distance value obtained, and shorter end-to-end distances are more heavily weighted in the averaging according to a R^{-6} dependence (see eq 2). We initially addressed this issue with the use of reagents that might alter the flexibility and thereby alter the average end-to-end distance. Measurements made in the presence of anti-Ig antibodies (Table II) or guanidine hydrochloride (Table III) do not provide a clear picture for the Ig structural dynamics. However, they are consistent with the view that IgE in solution is maintained in a bent structure (by noncovalent interactions), whereas IgG1 in solution has a greater amount of flexibility. These measurements have more general relevance because they clearly demonstrate how anti-Ig antibodies can cause significant perturbations of Ig structure. Similarly, guanidine hydrochloride at concentrations that do not affect antigen binding can cause significant structural changes.

The problem of extracting the distribution of distances between donor and acceptor probes as measured by energy transfer has been considered by others. Useful methods have been developed [e.g., Cantor and Pechukas (1971) and Haas et al. (1988)] that are based on the fact that the efficiency of energy transfer is maximal if the R_0 value for a given donor–acceptor pair is similar to their separation distance. Hence, different distance ranges are most sensitively measured by donor–acceptor probes with different R_0. We found the method developed by Gryczynski et al. (1988) to be well suited to our application. This group used collisional quenching as a means of altering the R_0 value for a single donor–acceptor pair and used steady-state fluorescence measurements to monitor the resulting change in donor–acceptor energy transfer. Energy transfer is measured as a function of quencher concentration, and the data are fit by a theoretical Gaussian distribution of distances parametrized by its average (ρ) and its half-width (hw). Their steady-state fluorescence analysis of model systems yielded results very similar to those obtained from higher resolution methods based on fluorescence lifetime decay measurements.

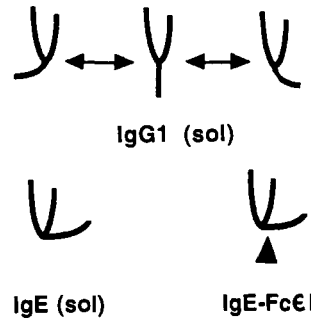

FIGURE 7: Structures and flexibilities are compared for IgE and IgG1 with a schematic model shown in perspective; the Fc C-terminal end is drawn to be equidistant from the two Fab N-terminal ends. IgE in solution (bottom left) and IgE bound to FcεRI on the plasma membrane (bottom right) appear to be similarly bent and relatively rigid; the arrowhead indicates the putative region of receptor interaction for IgE-FcεRI. IgG1 in solution (top) shows much greater flexibility than IgE. The distributions of distances (Figure 6) represented by these models are consistent with the view that IgE is asymmetrically bent on one side, whereas IgG1 flexes in more than one direction.

The distribution of distances we obtained for IgE in solution, IgE bound to FcεRI receptors on vesicles, and IgG1 in solution (Figure 6) are consistent with our single R_0 measurements (Table I), and they also yield new information about relative conformational flexibilities of these Ig. The distributions obtained provide the following view (Figure 7): IgE in solution is a bent molecule such that the distance between the ends of the Fab segments and the end of the Fc segment is, on average, 75 Å (=ρ); a limited amount of flexibility allows this distance to vary in the range of roughly 63–87 Å (hw = 24 Å). IgE bound to FcεRI receptors on RBL cell membranes appear to be slightly more bent with somewhat less flexibility with an average end-to-end distance of 71 Å within a rough range of 65–77 Å. IgG1 in solution shows much greater flexibility than IgE such that the corresponding distribution of distances is much broader, ranging roughly over 57–143 Å. Comparison of the structural dimension of these Ig[4] with their apparent end-to-end distance ranges suggests that the Fc of IgE is bent to one side, whereas the Fc of IgG1 can bend in more than one direction (Figure 7).

The χ^2 surfaces represented in Figure 5 indicate the resolution and level of uncertainty of the ρ and hw distance parameters determined with the analysis (eq 5). In all cases the range in which the minimum occurs is clear, although the breadth of the minimum varies (hence the uncertainty in the location of its absolute extremum). In general, the minima are sharper for the IgE samples, consistent with the result that the distance range recovered is closer to the range in which we were able to vary R_0[q] (45–50 Å). Our limitation to this relatively short range of R_0 values probably does not greatly affect the results. For their model system (R_0 = 30 Å), Gryzcynski et al. (1988) showed that distance parameters obtained for R_0[q] ranging from 10 to 30 Å were very similar to those ranging from 19 to 25 Å.

In Table I, the R values are always less than the ρ values. This is expected because the apparent distance R is more heavily weighted by the shorter distances (as discussed above), whereas for the ρ values each distance is weighted only by the probability of its occurring. If we take the distance distributions of Figure 6 and calculate the R value that would be

expected from eq 2, then the R_ρ values shown in Table I are obtained. The similarity between R and R_ρ for each of the samples demonstrates the overall agreement in the distance values obtained by these two independent sets of measurements.

Our structural interpretation of the energy-transfer results for these Ig is based on a simple model in which the acceptors are equally spaced from the donor such that only coordinate, symmetric flexing of the Fab segments with respect to the Fc segment occurs to yield a Gaussian distribution of end-to-end distances.[2] Structural information currently available from other physical measurements on these Ig does not allow us to evaluate the validity of this simple model in detail but does provide some insight. The reasonable (and common) assumption of symmetry is based on the X-ray-determined structure for crystalline IgG1, although only a hinge-deleted structure has been defined for an intact Ig. As discussed above, comparative fluorescence anisotropy studies of solution samples suggested that IgG1 has significantly more segmental flexibility than IgE, although the sources of this internal motion cannot be extracted unequivocally, and they are not necessarily symmetric. IgG1 motions could include twisting, wagging, and elbow bending of the Fab segments as well as wagging of the Fc segment. The more limited flexibility exhibited by IgE could include some restricted motion within the Fc segment; IgE bound to FcεRI receptors retains some relatively rapid internal motion which is likely to be the twisting of the Fab segments (Holowka et al., 1990). As pointed out by Gryzynski et al. (1988) and others who have developed distributional methods, the simple Gaussian function can be replaced by a more realistic function. However, the proper functional form for these Ig is probably very complex and is currently unknown. Despite their approximate nature the distributions provide useful information about the relative conformations and flexibilities of IgE and IgG. Furthermore, the ρ and hw values obtained by this independent method are consistent with the previous measurements indicating that IgG is more flexible than IgE and that receptor-bound IgE has a bent structure.

The conclusions drawn from these experimental data can be related to the known amino acid sequences within the Ig at the proposed regions of bending and flexing. Recently Helm et al. (1991) proposed a three-dimensional model of the human IgE Fc segment based on its known amino acid sequence, parallel disulfide bonds between the two heavy chains in the Cε2 domains, and structural homology with IgG1 for which the crystal structure has been determined. The eight residue stretch between the Cys^{328}–Cys^{328} disulfide bond in Cε2 and Val^{336}, which marks the start of Cε3, was modeled as an extended region of the polypeptide chain. This segment is included in a 76 amino acid fragment of IgE that was shown to compete effectively with intact IgE for binding to FcεRI (Helm et al., 1988). Although the eight-residue segment could be considered comparable to the "hinge" of IgG, it is possible that this segment has a bent structure that is not very flexible. Interestingly, IgG1 contains two adjacent glycines in its hinge segment compared to the single glycine in the IgE segment (Kabat et al., 1987). Because glycine allows a high degree of conformational freedom, this difference is consistent with the observation that IgG1 is more flexible than IgE. A detailed picture of the three-dimensional structure of the hinge regions may be derived from the amino acid sequence with energy minimization techniques (Vila et al., 1991). The results from our work indicate that the amino acids within this region or in adjacent domains stabilize a bent structure in IgE but not in IgG1.

In summary, this study provides a direct comparison of the conformations of IgE and IgG1, both in solution and when bound to membrane receptors. The results suggest that IgE in solution is bent in a relatively compact conformation that becomes even more rigid upon binding to its receptor. IgG1 appears to have a wider range of segmental motion that accounts for the short average distance between its ends in solution; restriction in these motions upon binding to receptor results in a longer average end-to-end distance.

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